PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/46

A1

(11) International Publication Number: WO 95/12408

(43) International Publication Date: 11 May 1995 (11.05.95)

(21) International Application Number: PCT/US94/12583

(22) International Filing Date: 3 November 1994 (03.11.94)

(30) Priority Data:
08/147,792 4 November 1993 (04.11.93) US

(71) Applicant: BRIGHAM & WOMEN'S HOSPITAL [US/US]; 75 Francis Street, Boston, MA 02115 (US).

(72) Inventors: SIMON, Daniel, I.; 211 Dorset Road, Waban, MA 02168 (US). LOSCALZO, Joseph; 16 Ledgewood Drive, Dover, MA 02030 (US).

(74) Agent: FREEMAN, John, W.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: PLASMIN-INDEPENDENT FIBRINOLYSIS

(57) Abstract

Disclosed is a method of dissolving thrombi in blood by introducing into the blood an aspartyl protease which cleaves fibrin or fibrinogen in blood clots.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

PLASMIN-INDEPENDENT FIBRINOLYSIS

Field of the Invention

This invention relates to the lysis of blood clots.

Background of the Invention

The fibrinolytic system, which dissolves blood clots and maintains blood flow at sites of vascular injury, is an important component of the normal hemostatic response. Endogenous fibrinolysis is achieved by the concerted actions of proteolytic enzymes, specific activators, and inhibitors of these proteases and activators. The major protease of the fibrinolytic system is plasmin.

The monocyte also plays an important role in regulating the thrombotic and fibrinolytic systems. Monocytes initiate the extrinsic pathway of coagulation by expressing mononuclear tissue factor, thus allowing the binding and activation of Factor VII on their surface. In turn, the prothrombinase complex assembles on the monocyte surface and generates thrombin. Monocytes also support fibrinolysis. The coordinated assembly of monocyte-secreted plasminogen activators and plasminogen on specific surface receptors enhances the catalytic efficiency of plasmin generation.

Cellular adhesion molecules are involved in cellular interactions which occur at the interface of thrombosis and fibrinolysis. These molecules regulate interactions between cells and between cells and matrix, and are believed to be important in the regulation of the immune system, inflammatory response, wound healing, as well as the pathophysiology of atherosclerosis.

One important class of adhesion molecules is that of the integrins. Integrins are transmembrane heterodimeric glycoproteins containing

noncovalently-linked alpha and beta subunits that exhibit the properties of functional versatility and ligand multispecificity. Mac-1 (CD11b/CD18) is a leukocyte integrin receptor that binds the heterogeneous ligands C3bi, Factor X, ICAM-1, and fibrin(ogen) (FGN) following activation with a variety of agonists, including adenosine 5'-diphosphate (ADP) and leukotriene B4. The binding of Mac-1 to ICAM-1 results in the adhesion of neutrophils and monocytes to the endothelium and after binding to Factor X, Mac-1 coordinates the activation of Factor X culminating in rapid fibrin formation.

Summary of the Invention

It has now been found that activated monocytes possess an alternative fibrinolytic pathway utilizing the cellular adhesion receptor Mac-1, which directly binds and internalizes FGN resulting in lysosomal degradation of FGN. This pathway of FGN degradation is plasmin-independent and mediated by a monocytic aspartyl protease, cathepsin D. This novel function of cathepsin D may have important therapeutic utility in the treatment of thrombotic disorders.

In general, the invention features a method of dissolving thrombi in blood by introducing into the blood an aspartyl protease which cleaves fibrin or fibrinogen in blood clots. By the term "aspartyl protease" is meant a proteolytic enzyme, the active site of which contains two aspartic acids. The protease may be a mixture of isozymes or a single isozyme, which may be monomeric or dimeric in nature and is capable of cleaving fibrin or fibrinogen at acidic pH. "Fibrinolysis" is defined as the cleavage and degradation of fibrinogen, fibrin or fibrin clots.

In one embodiment, the protease is cathepsin D. The invention also includes active fragments of cathepsin D. By "fragment" is meant a polypeptide ordinarily about

10 amino acids, typically about 20 amino acids, more typically about 30 amino acids, usually at least 40 amino acids, preferably at least 50 amino acids, and most preferably at least 60 to 80 or more contiguous amino acids in length. A fragment is "active" if it has at least 10%, more preferably at least 50%, and most preferably at least equal to the fibrinolytic activity of naturally occurring cathepsin D. In other embodiments, the protease is cathepsin E or an active fragment of cathepsin E. In yet another embodiment, the aspartyl protease is capable of inactivating an inhibitor of tissue plasminogen activator.

The invention also includes a method of dissolving blood clots in which an aspartyl protease is administered either simultaneously or sequentially with a plasminogen activator, such as tissue plasminogen activator or urokinase.

In another aspect, the invention features a method of preventing unwanted bleeding by introducing into the blood an inhibitor of aspartyl protease-mediated fibrinolysis, such as pepstatin A, diazoacetyl norleucine methyl ester, potato-derived cathepsin D inhibitor (PDI), phosphinate transition-state analog inhibitors, indomethacin, or flufenamic acid. By the term "inhibitor" is meant a substance which acts directly upon the aspartyl protease to reduce or abrogate its fibrinolytic activity. Also included in the invention is a method of preventing unwanted bleeding by introducing into the blood a compound which prevents FGN binding to Mac-1 on monocytes, such as a Mac-1 binding fragment of FGN.

In yet another aspect, the invention includes a method for screening candidate compounds to identify a compound capable of inhibiting aspartyl protease-mediated fibrinolysis. Such a screening assay involves providing

FGN in the presence and absence of the candidate compound, contacting FGN with an aspartyl protease, and determining the amount of fibrinolysis. A decrease in lysis in the presence of the candidate compound compared to that in its absence is an indication that the compound inhibits aspartyl protease-mediated fibrinolysis.

In a final aspect, the invention includes a screening method to identify a compound capable of inhibiting aspartyl protease-mediated fibrinolysis by monocytes. Such a screening assay involves providing a fibrin clot or FGN in the presence and absence of said candidate compound, contacting the clot or FGN with a Mac-1 expressing activated monocyte, and determining the amount of fibrinolysis. A decrease in lysis in the presence of the candidate compound compared to that in its absence is an indication that the compound inhibits aspartyl protease-mediated fibrinolysis by monocytes.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawing

Fig. 1 is a line graph showing the specific binding of [^{125}I]-FGN to U937 monocytoid cells at 4°C in the presence of 10 μM ADP, 2.5 mM $CaCl_2$, and 3 mM L-glycyl-L-prolyl-L arginyl-L-proline (GPRP). Each point represents the mean ± standard deviation of the number of molecules of FGN bound per cell (n = 4, each performed in duplicate).

Fig. 2 is a bar graph showing FGN and bovine serum albumin (BSA) degradation. The degradation of 0.9 μM [^{125}I]-FGN ± equimolar BSA or 0.9 μM [^{125}I]-BSA ± equimolar FGN by monocytoid cells at 37°C was evaluated. The molar ratio of protein degraded divided by FGN degraded was calculated. Each value represents the mean

± standard deviation (n = 3). [^{125}I]-FGN + BSA vs. [^{125}I]-BSA: p = 0.000028. [^{125}I]-BSA vs. [^{125}I]-BSA + FGN: p = 0.60.

Fig. 3 is a bar graph showing the effect of anti-integrin receptor monoclonal antibodies on FGN degradation by monocytoid cells. The degradation of FGN by monocytoid cells at 37°C was investigated in the presence of a monoclonal antibody, TS 1/18, directed to the beta-subunit (CD18) of Mac-1 that is also shared by LFA-1 and p150,95; monoclonal antibodies, LM2/1 and 2LPM19C, directed to the alpha-subunit (CD11b) of Mac-1; and as a negative control, monoclonal antibody, 7E3, directed to the platelet FGN receptor GPIIb/GPIIIa. Each value represents the mean ± standard deviation (n = 3-5).

Fig. 4 is an autoradiograph showing the proteolytic cleavage patterns of [^{125}I]-FGN exposed to U937 or THP-1 cells or to plasmin. Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) was performed under non-reducing conditions using a 7.5% polyacrylamide gel. Lane 1: [^{125}I]-FGN, Lane 2: [^{125}I]-FGN incubated with 1 CU/ml plasmin for 90 min. at 37°C, Lane 3: [^{125}I]-FGN supernatant exposed to ADP-activated THP-1 cells for 90 min. at 37°C, Lane 4: [^{125}I]-FGN supernatant exposed to ADP-activated THP-1 cells for 90 min. at 37°C, Lane 5: lysate of washed, ADP-activated U937 incubated with [^{125}I]-FGN for 90 min. at 37°C, Lane 6: lysate of washed, ADP-activated THP-1 cell incubated with [^{125}I]-FGN for 90 min. at 37°C.

Fig. 5 is a line graph showing the role of Mac-1 in fibrin clot lysis. A fibrin clot lysis assay was performed and percent clot lysis was calculated over time (n = 2). ● depicts the results of an experiment in which 1.0 nM tissue-type plasminogen activator (t-PA) added to THP-1 cell-enriched fibrin clot. o depicts the results of an experiment in which 1.0 nM t-PA added to fibrin

clot. Δ depicts the results of an experiment in which 40 μM D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (PPACK) added to THP-1 cell-enriched fibrin clot, and ▲ depicts the results of an experiment in which 40 μM PPACK + 0.035 mg/ml 2LPM19C was added to THP-1 cell-enriched fibrin clot.

Fig 6 is a bar graph showing the contribution of Mac-1-mediated fibrinolysis to total fibrinolysis with 1.0, and 3.2, nM t-PA. ADP-activated THP-1 cell fibrinolysis (■), t-PA-mediated fibrinolysis (□), and total fibrinolysis (⊠) were determined at 420 minutes. The results represent separate experiments performed in duplicate or triplicate.

Fig. 7 is a photograph showing immunolocalization of FGN in monocytoid cells. Immunofluorescence and immunoperoxidase microscopy of fixed, permeabilized monocytoid cells was performed in order to localize intracellular FGN. In panels "a" and "b", fluorescent-tagged anti-human FGN antibody was used to label cells. Panel "a" shows the surface binding of FGN to monocytoid cells. Binding was examined at 4°C in order to limit uptake and degradation of FGN. Panel "b" shows the results of experiments in which incubations were performed at 37°C in order to allow FGN uptake and degradation. Control incubations in which either FGN or the fluorescent-tagged antibody were omitted revealed no background fluorescence. Panel "c" shows immunoperoxidase staining of monocytoid cells after incubation at 37°C (400x magnification) and panel "d" shows monocytoid cells incubated with FGN and 420 nM factor X detected by immunoperoxidase staining (400x magnification).

Fig. 8 is a line graph showing the kinetics of the cleavage of FGN by cathepsin D. Varying concentrations of [^{125}I]-FGN (0.03 - 11.8 μM) were added to 70 nM

cathepsin D in 100 mM NaCl, 50 mM sodium acetate, pH 4.0 and the initial rates of proteolysis (v, μM/min) as a function of FGN concentration ([FGN], μM) were plotted.

Fig. 9 is a line graph showing the double reciprocal plot 1/v (min/μM) vs. 1/[FGN] (μM^{-1}) of the data shown in Fig. 8. The double reciprocal plot for the cleavage of FGN by 40 nM plasmin in TBS, pH 7.4, (▲) is included for comparison with cathepsin D (●). Each point represents the mean ± standard deviation of these experiments.

Fig. 10 is a line graph showing the pH profile of cathepsin D and plasmin fibrinogenolytic activity. The effect of pH on the rate of FGN proteolysis by cathepsin D and plasmin was investigated as described in Methods. Incubations were performed in 100 mM NaCl, 50 mM sodium acetate, pH 3.0-6.0. Incubations at pH 7.4 were performed in TBS. For cathepsin D (●), relative activity was calculated from the ratio of rate of FGN cleavage at the specified pH to the rate of cleavage at pH 3.5. For plasmin (O), relative activity was calculated from the ratio of rate of FGN cleavage at specified pH to the rate of cleavage at pH 7.4. Each value represents the mean of two experiments each performed in duplicate.

Fig. 11 is a line graph showing cathepsin D lysis of fibrin using the fibrin plate method. To a uniform fibrin layer, 20-μl samples containing varying concentrations of cathepsin D in 100 mM NaCl, 50 mM sodium acetate, pH 4.0 (▲); or t-PA in TBS, pH 7.4 (Δ); or buffer alone, 100 mM NaCl, 50 mM sodium acetate, pH 4.0 (●) applied to the fibrin layer and allowed to incubate at 37°C. After 18 hours, the zone of lysis (mm^2) was determined from the product of two perpendicular diameters. Zone of lysis was then plotted as a function of cathepsin D or t-PA concentration. Each value represents the mean ± standard deviation, n = 3.

Fig. 12 is a line graph showing the ability of cathepsin D to lyse fibrin clots. Fibrin clots were enriched with 11.5 mg/ml BSA, 10^7 red blood cells (RBC)/ml, and 11.5 mg/ml BSA/10^7 RBC/ml. To each fibrin clot, 400 nM cathepsin D in 100 mM NaCl, 50 mM sodium acetate, pH 4.0-6.0, was added containing 5 U/ml hirudin to inhibit residual thrombin activity and 40 μM PPACK to inhibit serine protease activity. Percent clot lysis was calculated over time for clots composed of fibrin (○), BSA-enriched fibrin (●), BSA/RBC-enriched fibrin (▲), or RBC-enriched-fibrin (Δ). Each value represents the mean ± standard deviation of these experiments.

Fig. 13 is a line graph showing rates of fibrin clot lysis affected by clot composition. The initial rates of clot lysis in fibrin (□), BSA-enriched fibrin (■), BSA/RBC-enriched fibrin (⊠), and RBC-enriched fibrin (□) clots were calculated from the initial slope of a plot of percent clot lysis versus time. The effect of pH on the rates of fibrin clot lysis was evaluated by adding cathepsin D in 100 mM NaCl, 50 mM sodium acetate, pH 4.0-6.0, to the fibrin clot formed at pH 7.4. Each value represents the mean ± standard deviation of these experiments.

Fig. 14 is a photograph of FGN and its proteolytic fragments generated by cathepsin D following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A terminal digestion of FGN by cathepsin D was performed by incubating plasminogen-free FGN with cathepsin D (molar ratio substrate to enzyme 1000:1) at 37°C for 18 hours. The terminal digest was first examined by performing SDS-PAGE using 12.5 % gels under reducing conditions.

Fig. 15 is a graph showing the results of high pressure liquid chromatography (HPLC) analysis of FGN fragments. To isolate FGN fragments for NH_2-terminal

sequence analysis, the cathepsin D-digested FGN was reduced, alkylated, and then subjected to HPLC using a reverse-phase column. Three major peaks were identified (A, B, C) and NH_2-terminal sequence analysis (8-15 cycles) was subsequently performed.

Description of the Preferred Embodiments

The invention features therapies using an aspartyl protease capable of cleaving fibrin or fibrinogen, and thus, capable of dissolving thrombi in blood. As is well known in the art, the class of proteases known as aspartyl proteases includes numerous known enzymes, such as pepsin, renin, yeast-derived proteinase A, and a mosquito lysosomal aspartyl protease which has been shown to bear homology to cathepsin D and E (Cho et al., 1992, *J. Biol. Chem.* 267:21823-21829). Fibrinolytic proteases, such as cathepsin D or E, can easily be identified using the procedures described below, e.g., solution-phase or fibrin plate assay to directly measure cleavage of FGN and the clot lysis assay to measure cleavage of FGN in blood clots.

Reagents

Human FGN (Grade L) and glu-plasminogen were purchased from Kabi/Pharmacia, Franklin, Ohio. Human plasminogen-free FGN was purchased from Enzyme Research Lab, South Bend, Indiana. Bovine spleen cathepsin D, E64D, pepstatin A, leupeptin, GPRP, N-methyoxysuccinyl-L-alanyl-L-prolyl-L-valanyl chloromethylketone (AAPVCK), and 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine (H7) were purchased from Sigma Co., St. Louis, Missouri. t-PA was obtained from Genentech Corp., South San Francisco, California and hirudin was obtained from Ciba-Geigy, Basel, Switzerland. Bovine thrombin was purchased from ICN BioMedical, Irvine, California. PPACK was purchased from Calbiochem Corp., La Jolla, California. Sepracell-

MN was purchased from Sepratech Corp, Oklahoma City, Oklahoma.

Monoclonal Antibodies

The antibody-producing hybridoma cell lines, TS 1/18 and LM2/1, were obtained from the American Type Culture Collection (ATCC) (Accession nos. HB203 and HB204, respectively). LPM19C was purchased from Dako Corp., Carpentiva, California.

Cells

The monoblast cell line, U937, and monocytic cell line, THP-1, were also obtained from ATCC (Accession nos. CRL1593 and TIB202, respectively). U937 and THP-1 cells were maintained in culture in RPM1 1640 containing 20% fetal calf serum, 20 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES), and 2 mM L-glutamine. Human monocytes were prepared from whole blood by continuous density gradient separation using Sepracell-MN. Equal volumes of whole blood anticoagulated with citrate-phosphate-dextrose (CPD) and Sepracell-MN were mixed and centrifuged at 1500 x g for 20 minutes at room temperature with a swinging bucket rotor. Peripheral blood mononuclear cells (PBMC), containing mostly lymphocytes and monocytes, are found in the compact opalescent band below the meniscus. Mononuclear cells were washed with 10 mM sodium phosphate, pH 7.4, 0.15 M NaCl (PBS) and 0.7% BSA mixed in one-and-one-half volumes of Sepracell-MN, and centrifuged at 1500 x g for 20 minutes. Monocyte-enriched mononuclear cells (greater than 85% monocytes) are found in the compact band below the meniscus, and lymphocyte-enriched mononuclear cells at the bottom of the tube. Cells were counted with a Coulter Counter, Model ZM (Coulter Immunology, Hialeah, FL).

Protein Labelling

Plasminogen-free human FGN and BSA were radiolabelled with [^{125}I] using Iodobeads (Pierce Chemical Company, Rockford, IL). The specific activity of [^{125}I]-FGN and [^{125}I]-BSA ranged from 3-5 x 10^4 cpm/μg and 1-2 x 10^4 cpm/μg, respectively; total radioactivity was greater than 95% trichloroacetic acid (TCA)-precipitable.

Fibrinogen Binding and Degradation Assays

The binding of [^{125}I]-FGN to monocytes was investigated as described by Altieri (Altieri et al., 1986, *J. Clin. Invest.* 78:968-976; Altieri et al., 1988, *J. Cell Biol.* 107:1893-1900, both of which are herein incorporated by reference) with the following modifications. U937 cells at 1.0 x 10^7/ml were suspended in TBS, pH 7.4, in the presence of 2.5 mM $CaCl_2$. Varying concentrations of [^{125}I]-FGN (0.1 - 4.0 μM) were added to a total volume of 250 μl and the cells stimulated with 10 μM ADP. The incubation was performed at 4°C in order to limit uptake and internalization of FGN. After 60 minutes, a 100-μl aliquot of the incubation mixture was layered onto 200 μl of silicone oil to separate free FGN from cell-bound FGN. Specific binding was calculated by subtracting nonspecific binding in the presence of a 20- to 50-fold molar excess of unlabelled FGN from total binding. Nonspecific binding accounted for 25-30% of total binding. Binding was also performed in the presence of 2.8 mM GPRP to limit fibrin polymerization.

The degradation of [^{125}I]-FGN, [^{125}I]-fibrin monomer (FM), or [^{125}I]-BSA by U937 cells, THP-1 cells, and human PBMC was examined by performing incubations at 37° C. [^{125}I]-FM was obtained by the addition of 1.0 U/ml bovine thrombin to [^{125}I]-FGN in the presence of 3 mM GPRP; after 15 minutes, 5 U/ml hirudin was added to inhibit residual thrombin. Degradation experiments were performed with 0.9 μM FGN, 0.9 μM FM, 0.9 μM BSA, or 0.9

μM FGN/0.9 μM BSA. After 120 minutes, 30 μl of ice cold TCA was added and the incubation mixture was centrifuged at 12,000 g for 4 minutes in an Eppendorf microfuge. An aliquot of the supernatant was then counted to determine the amount of acid-soluble, ether-inextractable radioactive material generated by the cells, i.e., [^{125}I]-labelled iodotyrosine-containing peptides. Nonspecific degradation was determined in the presence of a 20- to 50-fold molar excess of unlabelled FGN, FM, or BSA. Specific degradation (total - nonspecific) is expressed as μg of FGN, FM or BSA protein degraded/ 10^6 cells/hr.

The mechanism of FGN degradation was evaluated by performing incubations in the presence of the serine protease inhibitors, 40 μM D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (PPACK), 10 μg/ml soy bean trypsin inhibitor (SBTI), and 280 Kallikrein inhibitor unit (KIU)/ml aprotinin; the granulocyte elastase inhibitor, 50 μM N-AAPVCK; 400 μM chloroquine, an inhibitor of lysozomal degradation; 420 nM Factor X, a competitive ligand with FGN for the Mac-1 receptor; the cysteine protease inhibitors, 0.5 mM leupeptin, 50 μM z-L-phenylalanyl-L-alanyl-CHN_2; and the aspartyl protease inhibitor, 0.9 mM pepstatin A. In order to exclude inadequate intracellular concentrations, inhibitors were also added to monocytoid cells exposed briefly to 0.002 mg/ml saponin; after five minutes, saponin-exposed cells were washed and resuspended in serum-free RPMI. Greater than 90% of resuspended cells excluded trypan blue after such brief saponin exposure.

In order to characterize further the mechanism of FGN degradation, incubations were also performed in the presence of 0.04 mg/ml TS1/18; 0.09 mg/ml LM 2/1, a monoclonal antibody to the $alpha_M$-subunit of MAC-1 (CD11b); 0.07 mg/ml 2LPM19C, a monoclonal antibody to the

alpha$_M$-subunit of Mac-1 (CD11b) that blocks FGN binding; and as a negative control, 5 μg/ml 7E3, a monoclonal antibody that blocks FGN binding to platelet GPIIb/IIIa. Relative degradation was calculated from the ratio of the rate of degradation in the presence of inhibitor to the rate of degradation in the absence of inhibitor.

[^{125}I]-Fibrinogen Autoradiography

The proteolytic cleavage pattern of [^{125}I]-FGN exposed to U937 or THP-1 cells or to plasmin was investigated using autoradioagraphy. [^{125}I]-FGN was incubated with ADP-stimulated U937 or THP-1 cells in the presence of 280 KIU/ml aprotinin as described above. After two hours, the cells were collected by centrifugation at 800 x g. An aliquot of supernatant was then removed to examine free versus bound [^{125}I]-FGN. The pellet was washed three times with TBS, pH 7.4, and solubilized with sample buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, and 0.0025% bromophenol blue) for SDS-PAGE. [^{125}I]-FGN (2.5 mg/ml) was also digested with 1 casein unit (CU)/ml plasmin for 90 minutes at 37°C.

[^{125}I]-FGN, [^{125}I]-FGN supernatant exposed to U937 or THP-1 cells, cell lysate, or plasmin-digested [^{125}I]-FGN were individually boiled for five minutes in sample buffer and electrophoresed under non-reducing conditions on a 7.5% sodium dodecyl sulfate-polyacrylamide gel. The gel was dried and exposed to Kodak X-Omat AR film.

Clot Lysis Assay

The contribution of Mac-1-mediated fibrinolysis to overall clot lysis was investigated as follows. Fibrin clots were formed by the addition of bovine thrombin (1 U/ml) to 0.65 ml of platelet poor plasma (PPP) to which [^{125}I]-FGN (approximately 280,000 total counts) was added, in the presence and absence of approximately 1-2 x 10^6 ADP-activated THP-1 cells. The fibrin clots were

formed in 4-ml test tubes and, after 30 minutes, the tubes were centrifuged at 3000 rpm x 10 min. The residual supernatant was removed and the tubes were then counted to assess total [^{125}I]-FGN incorporation. To each fibrin clot 1.0 ml of PPP was added, containing 5 U/ml hirudin to inhibit residual thrombin activity, plus the following: 1) 40 μM PPACK to inhibit endogenous plasminogen activator/plasmin t-PA activity; 2) 1.0, 3.2, or 12.5 nM t-PA; 3) 1.0, 3.2, or 12.5 nM t-PA to a fibrin clot containing THP-1 cells; 4) 40 μM PPACK to a fibrin clot containing THP-1 cells; 5) 40 μM PPACK + 400 μM chloroquine to a fibrin clot containing THP-1 cells; and or 6) 40 μM PPACK + 0.035 mg/ml 2LPM19C (Mab to α subunit of Mac-1) to fibrin clot containing THP-1 cells. The test tubes were subsequently placed on a rocker and 50-μl aliquots of supernatant were removed at 30, 60, 120, 240, 300, and 420 minutes to assay released [^{125}I]-FGN counts. Percent clot lysis [(counts released/total counts in each tube) x 100] was calculated over time (each assay performed in duplicate). Nonspecific loss of counts (i.e., counts released by rocking alone in the presence of PPACK) was subtracted from each sample.

Immunolocalization of Fibrinogen

To localize the internalization and degradation of FGN morphologically, we used immunofluorescence, immunoperoxidase labelling techniques, and immunoelectron microscopy of fixed, permeabilized cells. The binding of FGN to U937 cells at 4°C and degradation of FGN at 37°C were performed as described above. An aliquot of cells was removed to make a thin smear on a microscopy slide. Slides were air dried, fixed and permeabilized as follows. Cells were fixed to the slides with 10 mM sodium periodate, 75 mM lysine, and 2% paraformaldehyde, pH 7.4. Endogenous peroxidase was inactivated by two

successive 10-minute incubations with 10 mM H_2O_2 and 10 mM NaN_3. The cells were permeabilized with 0.2 mg/ml saponin in PBS-0.1% BSA. For immunofluorescence experiments, a fluorescent-labelled anti-human FGN antibody was employed. Cells incubated in the absence of FGN showed no detectable background fluorescence. For immunoperoxidase experiments, the primary antibody consisted of a 1:100 dilution of rabbit anti-human FGN antibody in PBS-0.1% BSA, 0.1% Tween 20. After incubation with a primary antibody for 45 minutes, the cells were washed extensively with PBS and then incubated with a 1:500 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody for 45 min. After extensive washing of the secondary antibody, the horseradish peroxidase reaction product was developed with diaminobenzidine and H_2O_2. Controls consisted of cells incubated in the absence of FGN, deletion of the primary antibody, and incubation in the presence of Factor X, which was found to inhibit degradation by 90%.

Fibrinolytic Assays

The ability of cathepsin D or plasmin to cleave FGN was investigated initially in a solution-phase assay. Plasmin was obtained by the addition of t-PA to 9.5 μM glu-plasminogen for 1 hour at 37°C. Varying concentrations of [^{125}I]-FGN (0.03 - 11.8 μM) were added to 70 nM cathepsin D in 100 mM NaCl, 50 mM sodium acetate, pH 4.0, or 40 nM plasmin in TBS, pH 7.4, and allowed to incubate at 37°C. Aliquots were removed at 0, 60, and 120 minutes and ice cold TCA was added followed by centrifugation at 12,000 g for four minutes in an Eppendorf microfuge. An aliquot of the supernatant was counted to determine the amount of [^{125}I]-labelled iodotyrosine-containing peptides generated by cathepsin D or plasmin cleavage of FGN. The rates of proteolysis were determined as a function of [^{125}I]-FGN concentration

in order to derive the double reciprocal plot, 1/v (min/μM) vs. 1/[FGN] (μM^{-1}).

To investigate the effect of pH on the rate of FGN proteolysis, incubations with cathepsin D were also performed in 100 mM NaCl, 50 mM sodium acetate, pH 3.0 - 6.0. For cathepsin D, relative activity was calculated from the ratio of the rate of FGN cleavage at a specific pH to the rate of cleavage at pH 3.5, for plasmin, relative activity was calculated from the ratio of the rate of FGN cleavage at a specified pH to the rate of cleavage at pH 7.4.

Fibrin Plate Assay

The ability of cathepsin D to lyse fibrin was examined using the fibrin plate method as described by Astrup and Mullertz (Astrup et al, 1952, *Arch. Biochem. Biophys.* 40:346-351, herein incorporated by reference) with the following modification. A uniform fibrin layer coating the bottom of a nine-cm Petri dish was produced by adding 0.5 U/ml bovine thrombin to 10.45 ml of 1.0 mg/ml plasminogen-free human FGN or Kabi grade L human FGN in TBS, pH 7.4, containing 45 mM $CaCl_2$ (Kabi grade L FGN contains sufficient plasminogen for plasmin generation by the plasminogen activator, t-PA). The solution was briefly mixed and uniform polymerization was allowed to occur at 25°C on a level surface. After 30 minutes, 20-μl samples containing varying concentrations of cathepsin D (16 nM - 10 μM) in 100 mM NaCl, 50 mM sodium acetate, pH 4.0, or t-PA (16 nM - 2 μM) in TBS, pH 7.4, were carefully applied to the fibrin layer and allowed to incubate at 37°C. After 18 hours, the zone of lysis (mm^2) was determined from the product of two perpendicular diameters measured with calipers to the nearest 1 mm. The lysis zone size was then plotted as a function of cathepsin D or t-PA concentration.

Fibrin Clot Lysis Assay

The ability of cathepsin D to lyse fibrin clots was investigated as follows. Fibrin clots were formed by the addition of bovine thrombin (1 U/ml) to 0.65 ml of 2.0 mg/ml plasminogen-free FGN in TBS, pH 7.4, to which [^{125}I]-FGN (≈ 500,000 total counts) was added in the presence of 5 mM $CaCl_2$. BSA-enriched and BSA/red blood cell (RBC)-enriched fibrin clots were also formed by the addition of 11.5 mg/ml BSA and 10^7 RBC/ml. The fibrin clots were formed in 4 ml test tubes and, after 30 minutes, the tubes were centrifuged at 3000 rpm for 10 minutes. The residual supernatant was removed and the tubes counted to assess total [^{125}I]-FGN incorporation. To each fibrin clot, 1.0 ml of 100 nM NaCl, 50 nM sodium acetate, pH 4.0 - 6.0, was added containing 5 U/ml hirudin to inhibit residual thrombin activity, 40 μM PPACK to inhibit serine protease activity, and 400 nM cathepsin D. The test tubes were placed on a rocker and 50-μl aliquots of supernatant were removed over time to assay released [^{125}I]-FGN counts. Percent clot lysis [(counts released/total counts in each tube) x 100] was calculated over time. Nonspecific loss of counts (i.e., counts released by rocking alone) was subtracted from each sample.

Cleavage Patterns of FGN Proteolysis

The cleavage pattern of FGN proteolysis by cathepsin D was investigated using SDS-PAGE and HPLC. Plasminogen-free FGN was incubated with cathepsin D (molar ratio substrate to enzyme 5 - 2000:1) at 37°C. Aliquots were removed over time and the enzyme reaction was stopped by boiling for 5 minutes in sample buffer for electrophoresis containing 0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 0.0025% bromophenol blue, with or without 2% 2-mercaptoethanol. SDS-PAGE was performed on 7.5 - 12.5% linear gradient slab gels using

a discontinuous buffer system, and the gels were stained with Coomassie Brilliant Blue. FGN cleavage fragments were separated and analyzed by SDS-PAGE under reducing conditions.

To isolate FGN fragments for NH_2-terminal sequence analysis, a terminal cathepsin D digest of FGN was reduced with 45 mM dithiothreitol, alkylated with 100 mM iodoacetamide, and then subjected to HPLC using a reverse phase C18 column developed with acetonitrile in 0.06% trifluoracetic acid (0-100% over 70 minutes). Amino-terminal sequence analysis of the isolated proteolytic fragments was performed on an Applied Biosystem 477A Protein Sequencer (Applied Biosystems, Foster City, CA) with an on-line phenylthiohydantoin amino acid analyzer.

Binding and Degradation of FGN by Monocytoid Cells

The interaction of FGN with the monocytoid U937 cell line was first observed at 4°C in order to focus on the surface binding of FGN and, in particular, to investigate the internalization and degradation of FGN. As shown in Fig. 1, FGN binds to U937 cells in a specific, saturable manner. Stimulation with ADP was necessary to induce binding, which is calcium-dependent. Analysis of the binding data by the method of Scatchard revealed a single class of receptors with K_d = 1.8 μM and B_{max} = 1.6 x 10^5 FGN molecules bound/cell.

The degradation of internalized FGN and FM by monocytoid cells was also measured (see Table 1). Also shown in Table 1 is a comparison of the rate of FGN degradation in the more differentiated THP-1 monocyte cell line with the rate in human peripheral blood (monocyte-enriched) mononuclear cells (PBMCs).

TABLE 1

FIBRINOGEN AND FIBRIN MONOMER DEGRADATION BY ACTIVATED MONOCYTOID CELLS

Cell	μg FGN DEGRADED/ 10^6 CELLS/HR	μg FM DEGRADED/ 10^6 CELLS/HR
U937	0.37 ± 0.13	0.55 ± 0.03
THP-1	1.38 ± 0.02	1.20 ± 0.30
PBMC	2.10 ± 0.20	2.52 ± 0.18

Each value represents the mean ± standard deviation of μg FGN or FM degraded/10^6 cells/hour (n = 3-11).

Mechanism of FGN Degradation

To exclude a non-specific uptake and degradation mechanism, i.e., nonreceptor-mediated pinocytosis/phagocytosis, monocytoid cell degradation of FGN was compared to monocytoid cell degradation of BSA (Fig. 2). ADP-activated monocytoid cells degraded significantly less [^{125}I]-BSA compared to [^{125}I]-FGN, e.g., 0.11 ± 0.06 (mean ± SD) mole BSA degraded/mole FGN degraded. Furthermore, coincubation of [^{125}I]-BSA with FGN failed to increase the rate of BSA degradation (0.07 ± 0.09 mole BSA degraded/mole FGN degraded), suggesting that FGN does not stimulate generalized pinocytosis/phagocytosis in this system.

In order to elucidate the mechanism of FGN degradation by monocytoid cells, incubations were performed in the presence of potential inhibitors (see Table 2). We found that degradation was not significantly inhibited by the serine protease inhibitors, aprotinin and soybean trypsin inhibitor. Similarly, degradation was not inhibited by the tripeptide serine protease inhibitor, PPACK, or the specific elastase inhibitor, AAPVCK. This lack of inhibition by both high- and low-molecular-weight serine protease inhibitors and the low-molecular-weight elastase

inhibitor indicates that the degradation is not mediated by a released serine protease or the formation of a "protected pocket." All experiments were carried out in the absence of plasmin(ogen), and plasminogen-free FGN was used in all cases.

FGN degradation was found to be inhibited by chloroquine, an inhibitor of lysosomal proteolytic degradation. Factor X, a competitive ligand with FGN for the Mac-1 receptor, also significantly blocked degradation by 90 ± 15% (mean ± S.D.).

Monoclonal Antibodies to Mac-1 (CDIIb/CD18) Inhibit FGN Degradation by Monocytoid Cells

The receptor required for FGN degradation was identified using cell surface-specific monoclonal antibodies. The Mac-1 beta-subunit-specific monoclonal antibody, TS 1/18, partially inhibited FGN degradation (see Fig. 3). Since the alpha-subunit confers individual receptor identity and ligand specificity, the effect of alpha-subunit-specific monoclonal antibodies, LM2/1 and 2LPM19C, on FGN degradation was also investigated. As shown in Fig. 3, 2LPM19C blocked degradation by 90 ± 7%, indicating that Mac-1 is the receptor responsible for FGN degradation. Although 2LPM19C is known to block FGN binding to Mac-1, only selected epitopes of the alpha-subunit of Mac-1 appear to be involved in the degradation of FGN as evidenced by the lack of inhibition by LM 2/1. The monoclonal antibody 7E3, which is specific for the platelet surface integrin glycoprotein IIb/IIIa, did not affect the degradation of FGN by monocytes.

Proteolytic Cleavage of [^{125}I]-FGN

The proteolytic cleavage of FGN was investigated by employing [^{125}I]-FGN autoradiography. Fig. 4 demonstrates that U937 and THP-1 cells internalize and proteolyze FGN producing 50 - 60,000 MW proteolytic

fragments (lanes 5 and 6). This cleavage pattern is clearly different from that observed with plasmin (lane 2). The absence of [^{125}I]-FGN cleavage in the supernatant of U937/THP-1 exposed cells (lanes 3 and 4) provides additional evidence that this FGN degradation pathway requires internalization.

Mac-1-Dependent Fibrinolysis Contributes to Overall Clot Lysis

The contribution of Mac-1-mediated fibrinolysis to overall clot lysis was investigated. Fig. 5 shows that ADP-activated THP-1 cells (in the presence of 40 μM PPACK to inhibit the endogenous plasminogen activator t-PA and plasmin) lyse fibrin clots. This component of fibrin clot lysis is secondary to Mac-1 as evidenced by inhibition with the monoclonal antibody 2LPM19C. Fig. 6 illustrates the contribution of Mac-1-mediated fibrinolysis to total fibrinolysis over a range of t-PA concentrations. At 1.0 and 3.2 nM t-PA (physiologic concentration), Mac-1-mediated fibrinolysis accounted for 34% and 16%, respectively, of total fibrinolysis (t-PA-mediated + ADP-activated THP-1 cell-mediated).

FGN Degradation Does Not Require Protein Kinase C

The effect of the protein kinase C inhibitor, H7, an isoquinoline sulfonamide derivative, on FGN degradation by ADP-stimulated THP-1 cells was explored. H7 had a minimal effect on FGN degradation, inhibiting degradation by only 7 ± 11% (mean ± S.D., n = 4). Lack of inhibition of FGN degradation by H7, coupled with the finding that BSA is not significantly degraded, suggests a selective FGN degradation pathway involving Mac-1.

FGN Accumulates Intracellularly At 37°C

Immunofluorescence and immunoperoxidase microscopy of fixed, permeabilized cells was used to directly localize FGN. At 4°C, FGN bound to cell surface receptors (Fig. 6, panel "a"); at 37°C, FGN accumulated

intracellularly in monocytoid cells (Fig. 6, panels "b" and "c"), an observation confirmed by immunoelectron microscopy. The requirement for Mac-1 was demonstrated by the reduction in FGN peroxidase product when cells were coincubated with Factor X (see Fig. 6, panel "d").

Characterization of the Lysosomal Enzymes Responsible For Mac-1-Mediated Fibrinolysis

Table 2 shows that chloroquine inhibits FGN degradation by 75%, suggesting that a lysosomal pathway is involved. The lysosomal cysteinyl protease inhibitors, leupeptin, E64D, and z-phe-ala-CHN_2, failed to inhibit degradation. However, the aspartyl protease inhibitor, pepstatin A, inhibited FGN degradation by 32% (range 15-48%). Thus, an aspartyl protease is responsible for this fibrinolytic activity.

The degradation of FGN by monocytoid cells was investigated in the presence of the following potential inhibitors: 280 KIU/ml aprotinin, 10 μg/ml SBTI, 40 μM PPACK, 50 μM AAPVCK, 100 μM chloroquine, 400 nM Factor X, 10 μM z-phe-ala-CHN_2, 50 μM E64D, 0.5 mM leupeptin, and 0.9 mM pepstatin A. Relative degradation shown in Table 2 was calculated from the ratio of the rate of degradation in the presence of inhibitor to the rate of degradation in the absence of inhibitor. Each value represents the mean ± standard deviation (n = 3-5). * Indicates significant inhibition, $p < 0.01$.

TABLE 2

INHIBITORS OF FIBRINOGEN DEGRADATION

Inhibitor	Relative Degradation
Aprotinin	0.85 ± 0.16
SBTI	1.21 ± 0.10
PPACK	0.90 ± 0.26
AAPVCK	1.00 ± 0.06
Chloroquine	0.25 ± 0.12*
Factor X	0.10 ± 0.15*
z-phe-ala-CHN_2	1.17 ± 0.03
E64D	1.13 ± 0.24

Leupeptin	1.48 ± 0.12
Pepstatin A	0.68 ± 0.17*

Fibrinolysis By Cathepsin D

The ability of cathepsin D, a lysosomal aspartyl protease, to cleave [^{125}I]-FGN and generate non-TCA precipitable [^{125}I]-labelled-iodo-tyrosine peptides was investigated. Figs. 8 and 9 demonstrate that cathepsin D cleavage of FGN follows Michaelis-Menten kinetics with a Michaelis constant, K_m, of 1.5 μM, catalytic rate constant, k_{cat}, of 1.4 x 10^{-3} sec $^{-1}$ and, catalytic efficiency, k_{cat}/K_m, of 9.3 x 10^{-4} μM^{-1} sec^{-1}. Pepstatin A inhibits greater than 98% of this fibrinolytic activity while the serine protease inhibitor, PPACK, and cysteinyl protease inhibitor, leupeptin, do not inhibit the fibrinogenolytic activity of this cathepsin D preparation, ruling out any significant contribution by contaminant protease activities.

Table 3 shows a comparison of the fibrinogenolytic potential of cathepsin D to that of plasmin. Plasmin cleavage of FGN also follows Michaelis-Menten kinetics with a k_m of 7.3 μM, k_{cat} of 0.10 sec^{-1}, and catalytic efficiency, k_{cat}/K_m, of 1.4 x 10^{-2} $\mu M^{-1}sec^{-1}$. Therefore, at their respective pH optima, plasmin is 15-fold more efficient than cathepsin D in the cleavage of FGN.

TABLE 3

KINETIC CONSTANTS FOR THE CLEAVAGE OF FIBRINOGEN BY CATHEPSIN D AND PLASMIN

	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($\mu M^{-1}sec^{-1}$)
CATHEPSIN D	1.5	1.4 x 10^{-3}	9.3 x 10^{-4}
PLASMIN	7.3	0.10	1.4 X 10^{-2}

In the experiments discussed above, varying concentrations of [^{125}I]-FGN were added to 70 nM cathepsin D in 100 nM NaCl, 50 mM sodium acetate, pH 4.0, or 40 nM

plasmin in TBS, pH 7.4. Kinetic constants for the cleavage of fibrinogen by cathepsin D and plasmin were determined from double reciprocal plots, 1/v (min/μM) vs. 1/[FGN (μM^{-1}).

pH Dependence

Cathepsin D is an aspartyl protease with a pH optimum of 3.5 for hydrolysis of human hemoglobin (Ikeda et al., 1989, *Int J Biochem* 21:317-326). Fig. 10 shows the pH activity profiles of cathepsin D and plasmin for FGN. The pH optimum for cathepsin D is approximately 3.5 with 50% activity at pH 5.0 and 10% at pH 6.0. In contrast, plasmin has diminished proteolytic activity in the acidic pH range with 10% residual activity at pH 5.0 and 6% at pH 4.0.

Fibrinolysis By Cathepsin D

The ability of cathepsin D to lyse fibrin was investigated utilizing the fibrin plate assay. Fibrinolysis, as a function of cathepsin D concentration, is shown in Figure 11. Increasing fibrinolysis is evident over a dose range of 100 nM - 10 μM cathepsin D. For comparison to a known fibrinolytic agent, the responses to t-PA at pH 7.4 and pH 4.0 are also included. The fibrin layer was formed at pH 7.4 in all cases, and the fibrinolytic agent was added in 20 μl buffer at the specified pH. At pH 7.4, t-PA is approximately 100-fold more potent than cathepsin D; however, when layered onto the fibrin plate in a sample buffer at pH 4.0, t-PA expresses negligible fibrinolytic activity compared to cathepsin D.

The ability of cathepsin D to lyse fibrin clots, BSA-enriched fibrin clots, and BSA/RBC-enriched fibrin clots was also investigated. Clots were specifically enriched with RBC and BSA because both are present in *in vivo* thrombi and, furthermore, hemoglobin and albumin are

known substrates for cathepsin D cleavage. A typical time course for cathepsin D lysis of a fibrin clot is shown in Fig. 12. Forty percent lysis occurs by 420 minutes. The initial rates of clot lysis by cathepsin D, as a function of clot composition and pH, are depicted in Fig. 13. Fibrinolysis by cathepsin D is accelerated 2-4 fold in BSA-enriched fibrin clots. RBC-enriched fibrin clots are comparatively resistant to lysis by cathepsin D; however, in the presence of albumin, lysis of RBC-enriched fibrin clots occurs efficiently.

Characterization of Fibrinogen Cleavage Products

To identify the cleavage products of FGN generated by cathepsin D and to locate their cleavage sites, cathepsin D digestion products were separated using SDS-PAGE or HPLC and further analyzed using NH_2-terminal sequence analysis. A time course of proteolysis by cathepsin D is shown in Fig. 14. Cleavage of the α-, β- and γ-chains of FGN occurred rapidly with the generation of multiple lower-molecular-weight bands.

To characterize representative cleavage sites, a reverse phase-HPLC separation of a terminal digest of FGN was performed yielding three major peaks (A, B ,C) (see Fig. 15). These predominant peaks were subjected to NH_2-terminal sequence analysis identifying cleavage sites at residues 219-220 (A), 394-395 (B), and 521-522 (C) on the α-chain of FGN. In Table 4, the actual sequence obtained by sequence analysis is underlined and cleavage sites were localized according to the published amino acid sequence of human FGN (Thomas et al., 1989, *Biochem. J.* 259:905-907).

TABLE 4

211 PNLVPGNFK*SQLQKVPPEWKALTDMP 236	(SEQ ID NO:1)
386 PNDPNWGTF*EEVSGNVSPGTRREV 411	(SEQ ID NO:2)
511 PGFFSPMLGEF*VSETESRGSESGIFT 536	(SEQ ID NO:3)

Cleavage sites are indicated by an asterisk and proline residues appear in bold-face type (P). Residue 219-220 is contained within the interzonal region of the α-chain (residues 195-239) (Doolitle et al., 1979, *Nature* 280:464-468, herein incorporated by reference), a region which is susceptible to cleavage by several proteases including plasmin, trypsin, and thermolysin, and is thought to be relatively exposed and readily accessible to proteolytic attack (Takagi et al., 1975, *Biochemistry* 14:5149-5156). The cleavage site at residue 394-395 is contained within zone ZM (middle section) of the α-chain, a region which contains seven of the α-chain's 10 tryptophan residues and is relatively nonpolar. More than half the amino acids in this region are glycine, serine, proline, or threonine. This section of the α-chain is likely to be in a random coil conformation with regularly interspersed turns. The cleavage site at residue 521-522 is contained within the carboxy-terminal zone (residues 425-610).

Cathepsin D cleaves FGN and another protein, apoB-100 (Van Der Westhuyzen et al., 1980, *Eur. J. Biochem.* 112:153-160) in proline-rich domains. However, cathepsin D cleavage sites of FGN do not conform to the common sequence pattern of "hydrophobic-charged-x-x-charged" reported for cathepsin D cleavage of other select cathepsin D substrates (van Noort et al., 1989, *J. Biol. Chem.* 264:14159-14164).

Mac-1 and Fibrin(ogen) Internalization by the Monocyte/Macrophage

The novel pathway disclosed herein involves a two-step mechanism in which Mac-1 on activated monocytoid cells first binds FGN, followed by the internalization and cathepsin D-mediated lysosomal degradation of FGN. FGN is intracellularly localized in monocytoid cells, a process that is blocked by factor X, which competitively

inhibits FGN binding to Mac-1. Mac-1 may also activate factor X culminating in rapid fibrin formation, and, thus, may play a role in modulating fibrin formation/dissolution.

The receptor for FGN on monocytoid cells is inducible and of relatively low affinity (K_d = 1.8 μM) with approximately 1 x 10^5 receptors present per cell was identified. Stimulation with a variety of agonists, such as ADP or leukotriene B4, is necessary to induce binding, which is calcium dependent.

Since monocytic cells can bind FGN and actively phagocytize bound ligands, the ability of human monocytes to degrade FGN was examined by performing incubations at 37°C and assaying the supernatant of TCA-treated cell suspensions for acid-soluble, ether-inextractable radioactive material generated by the cells. U937 cells, THP1 cells, and human PBMCs were found to degrade FGN and FM at rates of 0.37, 1.3, 2.1 μg FGN/10^6 cells/hr and 0.55, 1.20, 2.52 μg FM/10^6 cells/hr, respectively.

Binding of integrin receptors to their ligands may trigger signals which stimulate phagocytosis. Specifically, Gresham and coworkers (Ikeda et al., *supra*) have shown that adhesive proteins containing the Arg-Gly-Asp (RGD) sequence stimulated neutrophil erythrophagocytosis. However, contrast to the FGN uptake mechanism disclosed herein, the addition of catalase to inactivate products of the myeloperoxidase-hydrogen peroxide-halide system was required to demonstrate stimulated phagocytosis.

Disclosed herein is the first demonstration of direct uptake and degradation of a CD11b/CD18-bound ligand, FGN. Three pieces of evidence suggest that FGN degradation occurs by way of a selective mechanism and not by generalized pinocytosis/phagocytosis: 1) the degradation of [^{125}I]-BSA coincubated with FGN is

comparatively negligible (see Fig. 2); 2) FGN degradation is unaffected by the protein kinase C inhibitor H7, which inhibits stimulated erythrophagocytosis; and 3) FGN degradation does not require the addition of catalase.

Example 1: Fibrinolytic Therapy

The invention provides novel therapeutic approaches to the treatment of thrombotic disorders.

Although a highly specialized fibrinolytic response, mediated by plasminogen activator/plasmin, has evolved to limit thrombus formation to areas of vascular injury, unchecked intravascular thrombosis can occur, and in some cases, is the precipitating event in the clinical syndromes of unstable angina and myocardial infarction. Furthermore, fibrin(ogen) degradation products are capable of accumulating in the vessel wall leading to smooth muscle proliferation, endothelial cell injury or dysfunction, and increased vascular permeability. Other thrombogenic situations, include but are not limited to deep venous thrombosis, pulmonary embolus, occluded arterio-venous shunts, peripheral arterial occlusion, regression of atherosclerosis, and occluded in-dwelling or permanent catheters, e.g., those used for chemotherapy.

Numerous pathologic studies have documented the accumulation of FGN and fibrin(ogen)-related degradation products prior to the development of atherosclerosis. FGN and its degradation products may affect the atherogenic process by virtue of their ability to (1) stimulate vascular smooth muscle cell proliferation, (2) alter endothelial cell permeability, (3) inhibit prostacyclin production, and (4) inhibit t-PA production. Therapeutic administration of cathepsin D may be useful in clearing FGN and its degradation products.

Since local regions of hyperacidity can form during inflammation as cells switch to glycolysis and secrete lactic acid resulting in pH of 3.5 to 5.0, the fibrinolytic proteases of the invention are ideal candidates for therapeutic intervention. In such situations, cathepsin D can be used to clear FGN/fibrin in areas of inflammation and ischemia where plasminogen activator/plasmin activity is limited by acidic pH. Administration of a fibrinolytic protease of the invention with another fibrinolytic agents, such as streptokinase, staphylokinase, urokinase, or t-PA would then be appropriate. These agents can be administered to a patient sequentially or simultaneously.

Fibrinolytic proteases, such as cathepsin D and E can be purified from tissue, e.g., spleen or liver tissue using known methods, e.g., Takahashi et al., 1981, *Meth. Enzymol.* 80:565-581; Yamamoto et al., 1978, *Eur. J. Biochem.* 92499-508, both of which are herein incorporated by reference. Alternatively, cDNA encoding the fibrinolytic protease, e.g. cathepsin D (Faust et al., 1985, Proc. Natl. Acad. Sci USA 82:4910-4914, herein incorporated by reference), e.g., cathepsin E (Tsukuba et al., 1993, *J. Biol. Chem.* 268:7276-7282; Hill et al., 1993, *FEBS Letters* 326:101-104, both of which are herein incorporated by reference) can be cloned into an expression vector and produced by a procaryotic or eucaryotic cell using methods well known in the art of molecular biology. The protease can then be purified using methods described above or any number of protein purification procedures known in the art, e.g., Pepstatin-Sepharose (Sigma, St. Louis, MO) or immunoaffinity chromatography using an antibody specific for the gene product to be purified, e.g., anti-cathepsin D antibody (Calbiochem, La Jolla, CA) for purification of cathepsin D.

Following purification, the fibrinolytic proteases of the invention can be administered to patients in a pharmaceutically acceptable carrier such as physiological saline, in a manner similar to those presently used for the administration of streptokinase or t-PA. Fibrinolytic proteases can be administered intraperitoneally, intramuscularly, subcutaneously, or intravenously. It is expected that the preferred route of administration is intravenous. Fibrinolytic proteases can be administered systemically as well as locally within the blood vessel at the site of clot formation.

As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages for the fibrinolytic proteases of the invention will vary, but a preferred dosage for intravenous administration is approximately 10-1000 μmoles/kg. For local infusion, it is expected that the dose will be 4-5 times that the dose administered systemically.

Fibrinolytic proteases, such as cathepsin D, are particularly useful because of their ability to inhibit an endogenous inhibitor of t-PA (PAI-1), thus allowing maximal plasmin-mediated fibrinolytic activity.

The potential limitation of cathepsin D activity at pHs greater than 6 may be possible to overcome by the liposomal delivery of cathepsin D. Using methods known in the art to select the appropriate phospholipids, liposomes can be constructed with a sufficiently acidic pH to preserve cathepsin D activity.

The invention also includes an *ex vivo* method of therapy. This method of the invention would be of particular benefit in situations in which the blood of a

patient is removed for filtering (e.g., kidney dialysis) or gas exchange procedures, or when the patient requires blood transfusions. For treatment of blood extracorporeally, blood can be removed from the individual using methods known to those skilled in the art, such as venous puncture. The fibrinolytic protease, in a physiologically acceptable carrier, can then be mixed with the blood, and subsequently returned to the individual using methods known to those skilled in the art, such as intravenous drip.

The methods of the invention offer many therapeutic advantages. As discussed above, cathepsin D is active at acid pH. In addition, cathepsin D cleaves and thus, inactivates PAI-1, the physiological inhibitor of the endogenous plasminogen activator, t-PA. These properties confer three distinct advantages for the use of cathepsin D for fibrinolytic therapy: (1) cathepsin D is fibrinolytically active in areas of ischemia where the pH has been shown to be less than 5.5; (2) cathepsin D cleavage of PAI-1 attenuates the inhibition of t-PA, thereby favoring full endogenous fibrinolytic potential; and (3) relative fibrin/clot specificity by virtue of the favorable pH-activity profile of cathepsin D with negligible enzymatic activity in plasma at pH 7.4.

In addition, cathepsin D activity is stimulated by phospholipids, suggesting that platelet-rich or cell-rich thrombi are potentially capable of enhancing cathepsin D activity. This property may confer an additional advantage to cathepsin D over t-PA, which has reduced activity in platelet-rich thrombi.

In some cases, it is advantageous to inhibit endogenous fibrinolysis to prevent unwanted or excessive bleeding. For example, 5-10% of patients who have undergone cardiopulmonary bypass surgery develop bleeding disorders. In such cases, cardiopulmonary bypass leads

to the activation of monocytes (Gu et al., 1992, Ann. Thorac. Surg. 53:839) which in turn, leads to the induction of cell surface expression of Mac-1. Activated Mac-1-expressing monocytes at the site of tissue injury may contribute to excessive bleeding by degrading FGN.

Administration of compounds which inhibit the enzymatic activity of aspartyl protease, e.g., pepstatin A, diazoacetyl norleucine methyl ester, PDI (Ritonja et al., 1990, FEBS Letters 267:13-15), phosphinate transition-state analog inhibitors (1990, Biochem. Biophys. Res. Comm. 169:1111), indomethacin, or flufenamic acid (Yamamoto, K. et al., 1988, Nippon Yakurigaka Zashii 91:371-376), can prevent monocyte-mediated FGN degradation, thus allowing clot formation in patients with such bleeding disorders. Since aspartyl protease-mediated fibrinolysis *in vivo* is likely to occur within monocytic cells, inhibitors may be packaged in liposomes or microcapsules, using methods known in the art, to assure delivery of the inhibitor into the cell. Some aspartyl protease inhibitors, e.g., phosphinate transition-state analog inhibitors, indomethacin, flufenamic acid, and diazoacetyl norleucine methyl ester, can readily gain access to the cytoplasm of the cell and thus, can be administered without the benefit of such delivery systems.

Monocyte-mediated fibrinolysis may also contribute to other types of bleeding disorders, such as familial hemophagocytic lymphohistiocytosis (FHL) which is characterized by hypofibrinogenemia (Ooe, K., 1991, Ped. Path. 11:657-661). Monocyte-mediated fibrinolysis may be inhibited by administering a compound which prevents binding of FGN to the Mac-1 receptor on the cell surface, e.g., a Mac-1 binding fragment of FGN, thus preventing cellular internalization and degradation of FGN.

Example 2: Screening of candidate compounds

To identify aspartyl proteases with fibrinolytic activity, the assays described above, e.g., solution phase fibrinolytic assay, fibrin plate assay, and fibrin clot assay, can be used to measure clot lysis or FGN cleavage in the presence and absence of the candidate protease. Fibrinolysis observed in the presence of the candidate protease compared to a negative control or absence of the candidate protease indicates that the candidate protease has fibrinolytic activity. A protease with known fibrinolytic activity, such as cathepsin D, can be used as a positive control.

To identify inhibitors of aspartyl proteases, the assays described above, e.g., solution phase fibrinolytic assay, fibrin plate assay, and fibrin clot assay, can be used to measure aspartyl protease-mediated fibrinolysis in the presence and absence of a candidate compound. In this case, a FGN can be incubated with the candidate compound and then contacted with an aspartyl protease, such as cathepsin D or E. Alternatively, the aspartyl protease can first be incubated with the candidate compound and then allowed to contact FGN. Fibrinolysis is then measured by any of the assays described above. A decrease in lysis in the presence of the candidate compound compared to lysis in its absence indicates that the candidate compound inhibits aspartyl protease-mediated fibrinolysis.

Similarly, compounds capable of inhibiting aspartyl protease-mediated fibrinolysis by monocytes can be identified using the clot lysis assay or FGN degradation assay described above. In this modified assay, a preformed clot or FGN is incubated with a candidate compound, contacted with activated monocytes, e.g., ADP-activated THP-1 or U937 cells, and fibrinolysis evaluated. Alternatively, the activated monocytes may be

incubated with the candidate compound prior to contact with the preformed clot or FGN. As above, a decrease in fibrinolysis in the presence of the candidate compound compared to lysis in its absence indicates that the candidate compound inhibits aspartyl protease-mediated fibrinolysis.

Example 3: *In vivo* fibrinolytic properties of cathepsin D

The fibrinolytic potential of cathepsin D *in vivo* was investigated using the rabbit jugular vein model (Collen et al., 1983, J. Clin. Invest. 71:368, herein incorporated by reference). Human FGN was radioiodinated using Iodobeads, as described above. The radiolabelled FGN routinely had a specific activity of approximately 75,000 cpm/μg. Each rabbit, weighing 2-4 kg, was anesthetized with ketamine HCl (0.5-1.0 g/kg body weight) during the procedure. The right external jugular vein was located by direct exposure while preserving its two major branches: the facial vein and the more distal (superior) posterior occipital branch. All other small branches were carefully ligated with 4-0 silk suture to prevent possible leakage. The jugular vein was occluded with a 2-0 silk suture proximately and just distally to the branch point of the facial vein. the facial vein is then cannulated with 0.038 inch polyethylene tubing filled with normal saline. The isolated jugular vein was then emptied of blood.

A 500 μl aliquot of blood, drawn from the femoral vein, was mixed with a 10 μl sample of [^{125}I]-FGN (approximately 500,000 cpm), followed by the addition of 10 μl of bovine thrombin (200 U/ml). The sample was then immediately injected into the jugular vein via the facial vein cannula, and the clot allowed to incubate for 30 minutes. During the incubation, the more distal posterior occipital branch was cannulated. After this incubation period, both the jugular vein ties were

removed leaving a thrombosed segment of jugular vein adjacent to the facial vein and a more distal (superior) branch cannulated for local infusion.

Rabbits were treated by venous infusion of cathepsin D. One ml of 8 μM cathepsin D in 50 mM sodium acetate, 100 mM NaCl, pH 4.5, was diluted in normal saline to a total volume of 2 ml and administered over 60 minutes. For control rabbits, the infusion consisted of acetate buffer alone diluted in normal saline. After 120 minutes from the start of infusion, the ligatures were re-tied and the segment of jugular vein at the site of thrombosis excised. The syringe, needle, and cannula used to inject the [^{125}I]-FGN containing blood sample were saved along with all swabs used to clean the surgical field. The radioactivity of the jugular vein segment was measured (cpm) and percent clot lysis calculated after subtracting the radioactivity of the various sources previously listed from the initial starting dose of [^{125}I]-FGN to obtain the total cpm injected for clot formation.

Profound *in vivo* fibrinolytic activity was seen at the site of a thrombus when cathepsin D was intravenously infused into rabbits (see Table 5). Cathepsin D infusion resulted in approximately 80% clot lysis over two hours.

TABLE 5

In Vivo Fibrinolysis by Cathepsin D

Infusion	Total clot ^{125}I (cpm)	Residual clot ^{125}I (cpm)	%Lysis
Control	383,900	430,200	0
Cathepsin D	437,300	84,700	81

Other embodiments:

For prevention of thrombotic occlusion post-operatively, a bio-polymer delivery system designed for the slow release of the fibrinolytic protease of the invention may be implanted in close proximity to blood vessels that have been injured, such as those involved in coronary bypass surgery or coronary stent implantation. Such bio-polymer delivery systems are well known in the art (see, e.g., Folkman et al., U.S. Patent 4,164,560, herein incorporated by reference). The stent itself can be made of a bio-polymer that has been impregnated with the fibrinolytic protease of the invention, and which therefore mediates slow local release of the protease to clear occlusion of the blood vessel.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Daniel I. Simon
Joseph Loscalzo

(ii) TITLE OF INVENTION: PLASMIN-INDEPENDENT FIBRINOLYSIS

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Fish & Richardson
(B) STREET: 225 Franklin Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: U.S.A.
(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM: MS-DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE: 03 November 1994
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 08/147,792
(B) FILING DATE: 04 November 1993

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: John W. Freeman, Esq.
(B) REGISTRATION NUMBER: 29,066
(C) REFERENCE/DOCKET NUMBER: 05311/006001

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 542-5070
(B) TELEFAX: (617) 542-8906
(C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Pro Asn Leu Val Pro Gly Asn Phe Lys Ser Gln Leu Gln Lys Val Pro
1 5 10 15

Pro Glu Trp Lys Ala Leu Thr Asp Met Pro
20 25

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Pro Asn Asp Pro Asn Trp Gly Thr Phe Glu Glu Val Ser Gly Asn Val
1 5 10 15

Ser Pro Gly Thr Arg Arg Glu Val
20

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Pro Gly Phe Phe Ser Pro Met Leu Gly Glu Phe Val Ser Glu Thr Glu
1 5 10 15

Ser Arg Gly Ser Glu Ser Gly Ile Phe Thr
20 25

What is claimed is:

1. A method of dissolving thrombi in blood, comprising introducing into said blood an aspartyl protease, wherein said protease cleaves fibrin or fibrinogen in said clots.

2. The method of claim 1, wherein said protease is capable of cleaving fibrin or fibrinogen at acid pH.

3. The method of claim 1, wherein said protease is cathepsin D.

4. The method of claim 1, wherein said protease is an active fragment of cathepsin D.

5. The method of claim 1, wherein said protease inactivates an inhibitor of tissue plasminogen activator.

6. The method of claim 1, further comprising introducing into said blood a plasminogen activator.

7. The method of claim 6, wherein said plasminogen activator is tissue plasminogen activator or urokinase.

8. The method of claim 1 or claim 5, wherein said aspartyl protease is administered to the bloodstream of a human patient to dissolve thrombi in said patient's blood.

9. A method of preventing unwanted bleeding comprising introducing into the blood an inhibitor of aspartyl protease-mediated fibrinolysis by monocytes.

10. The method of claim 9, wherein said inhibitor is pepstatin A, diazoacetyl norleucine methyl ester,

potato-derived cathepsin inhibitor, phosphinate transition-state analog inhibitors, indomethacin, or flufenamic acid.

11. A method of preventing unwanted bleeding comprising introducing into the blood a Mac-1-binding fragment of fibrin or fibrinogen.

12. A method for screening candidate compounds to identify a compound capable of inhibiting aspartyl protease-mediated fibrinolysis by monocytes comprising the steps of:

(a) providing a fibrin or a fibrin clot in the presence and absence of said candidate compound;

(b) contacting said fibrin or a fibrin clot with a Mac-1 expressing activated monocyte; and

(c) determining the amount of fibrinolysis, wherein a decrease in lysis in the presence of said candidate compound compared to that in the absence of said candidate compound is an indication that said compound inhibits aspartyl protease-mediated fibrinolysis by monocytes.

13. A method for screening candidate compounds to identify a compound capable of inhibiting aspartyl protease-mediated fibrinolysis comprising the steps of:

(a) providing fibrin or fibrinogen in the presence and absence of said candidate compound;

(b) contacting said fibrin or fibrinogen with an aspartyl protease; and

(c) determining the amount of fibrinolysis, wherein a decrease in lysis in the presence of said candidate compound compared to that in the absence of said candidate compound is an indication that said compound inhibits aspartyl protease-mediated fibrinolysis.

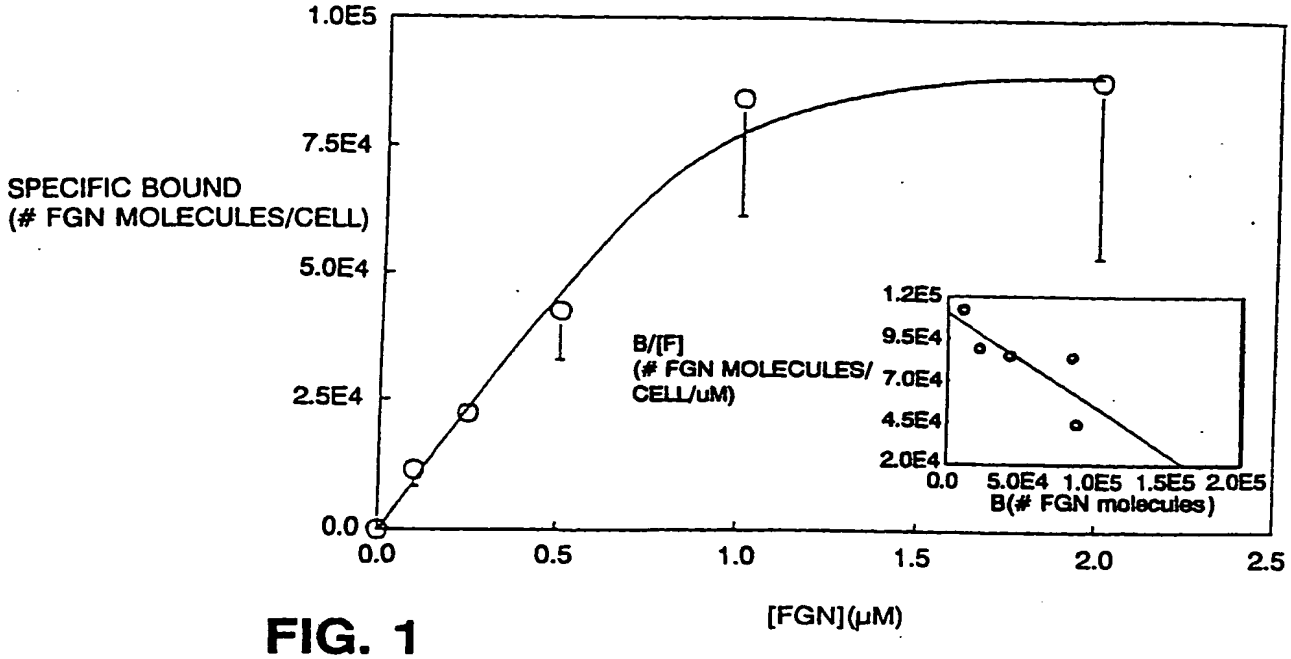

FIG. 1

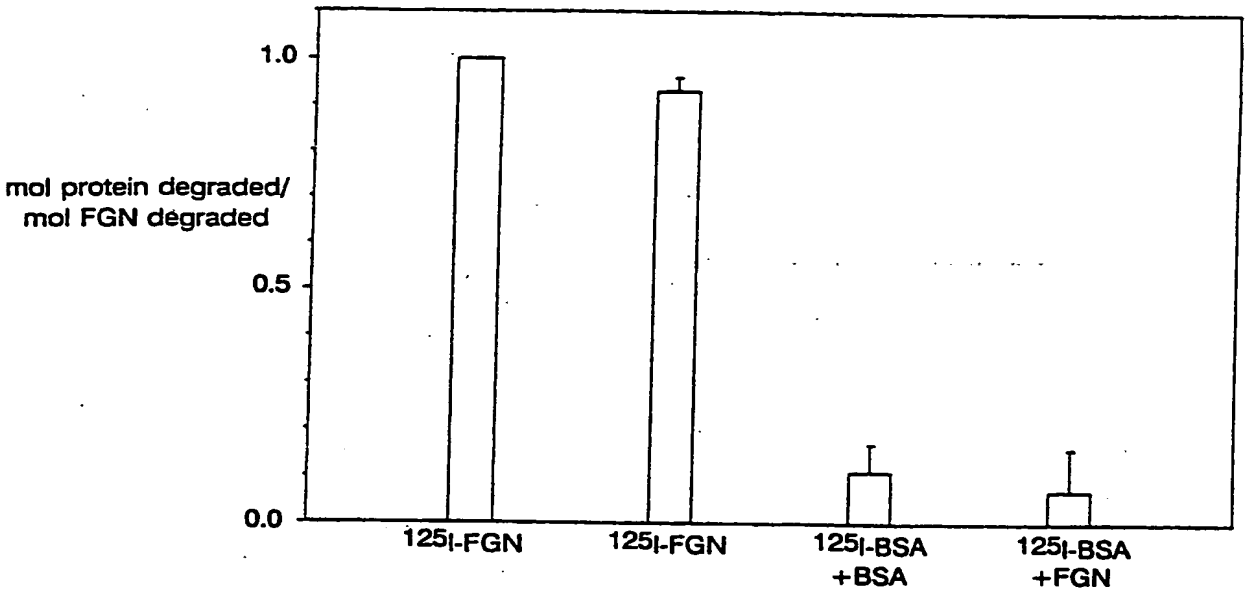

FIG. 2

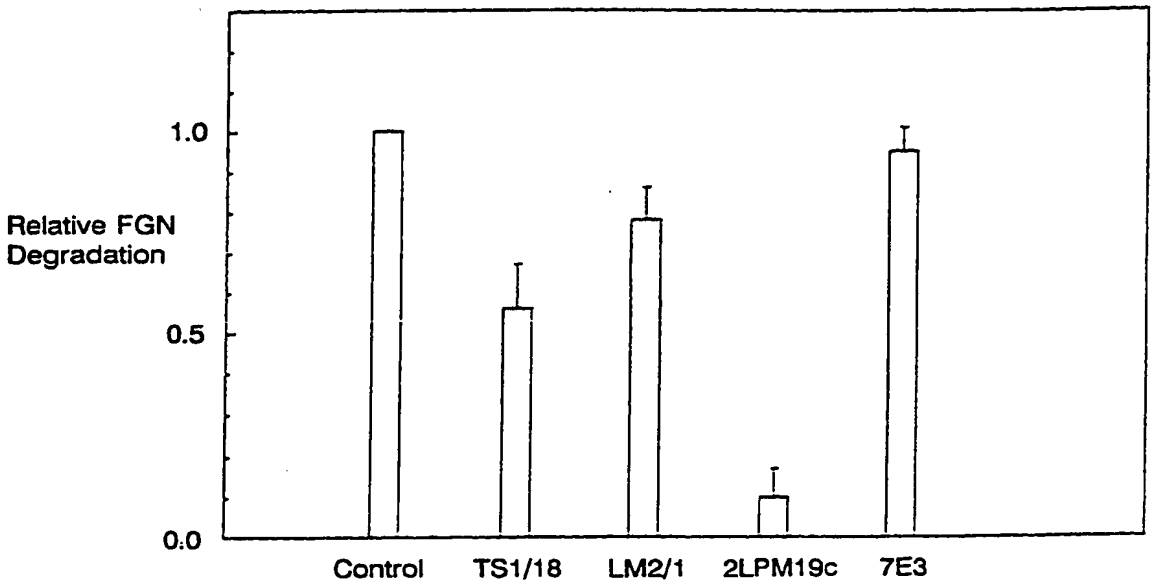

FIG. 3

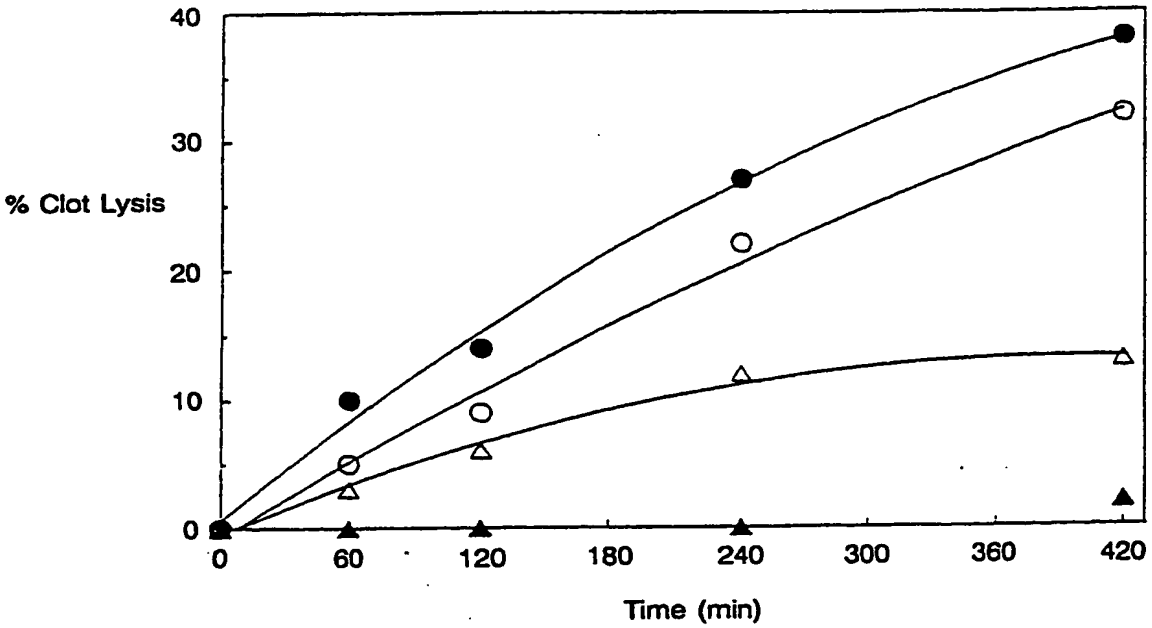

FIG. 5

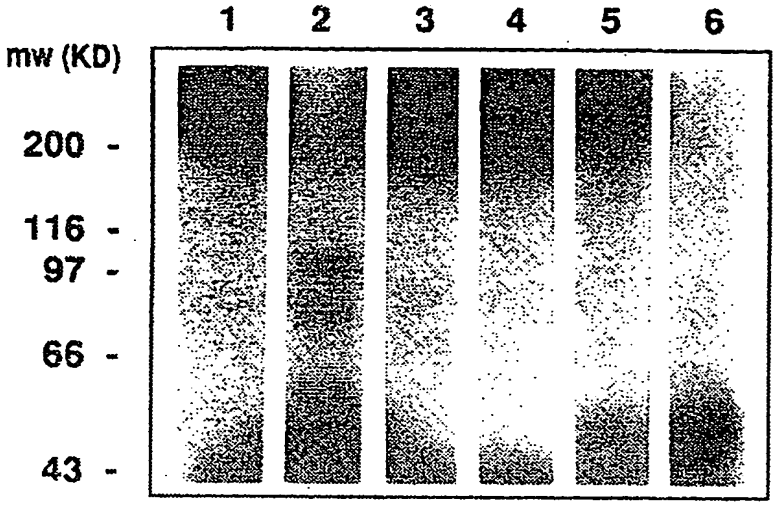

FIG. 4

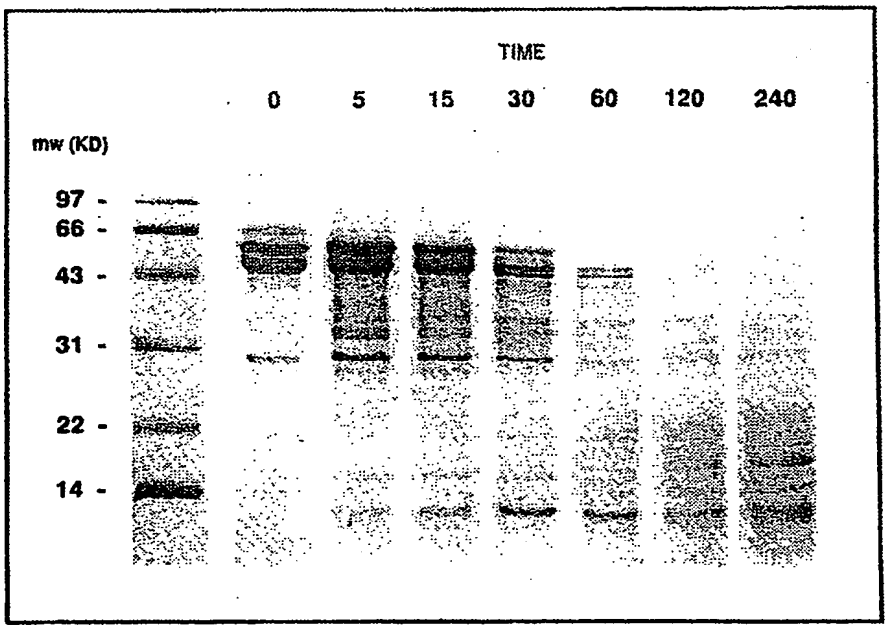

FIG. 14

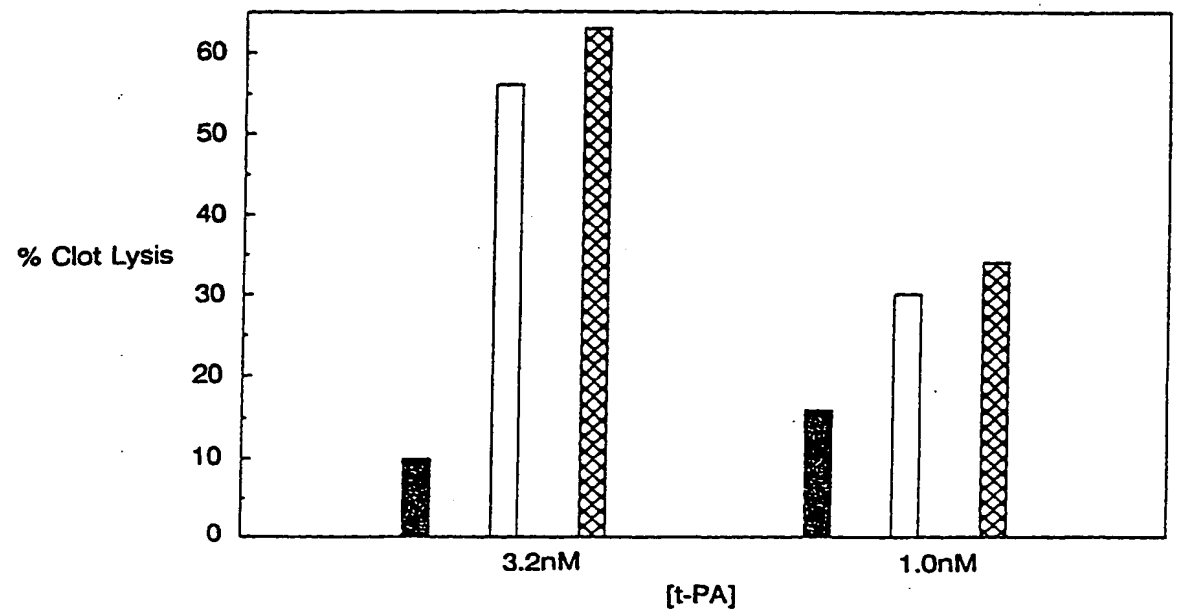

FIG. 6

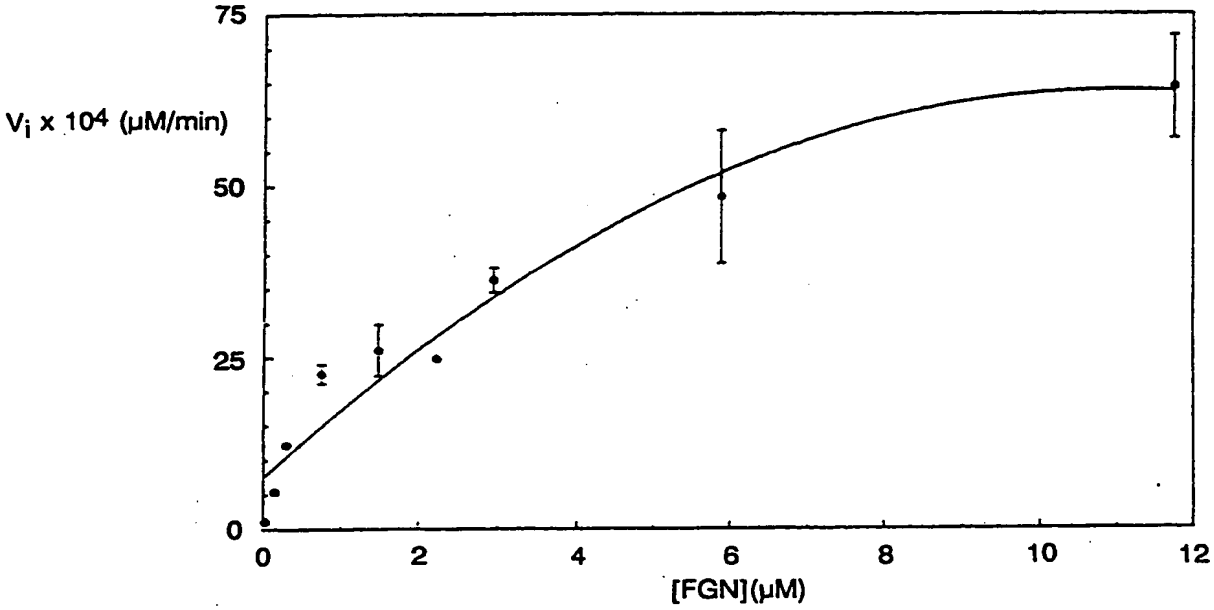

FIG. 8

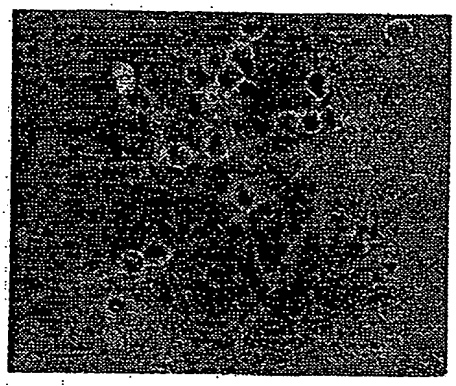

FIG. 7A

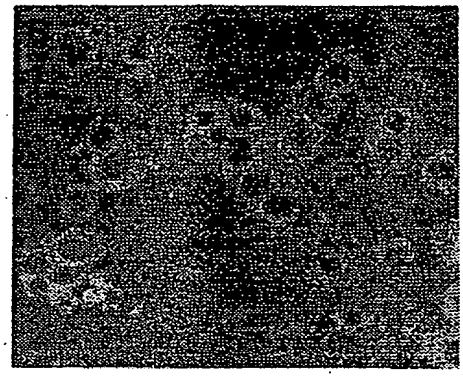

FIG. 7B

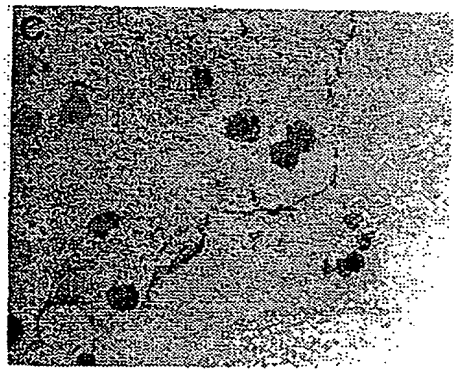

FIG. 7C

FIG. 7D

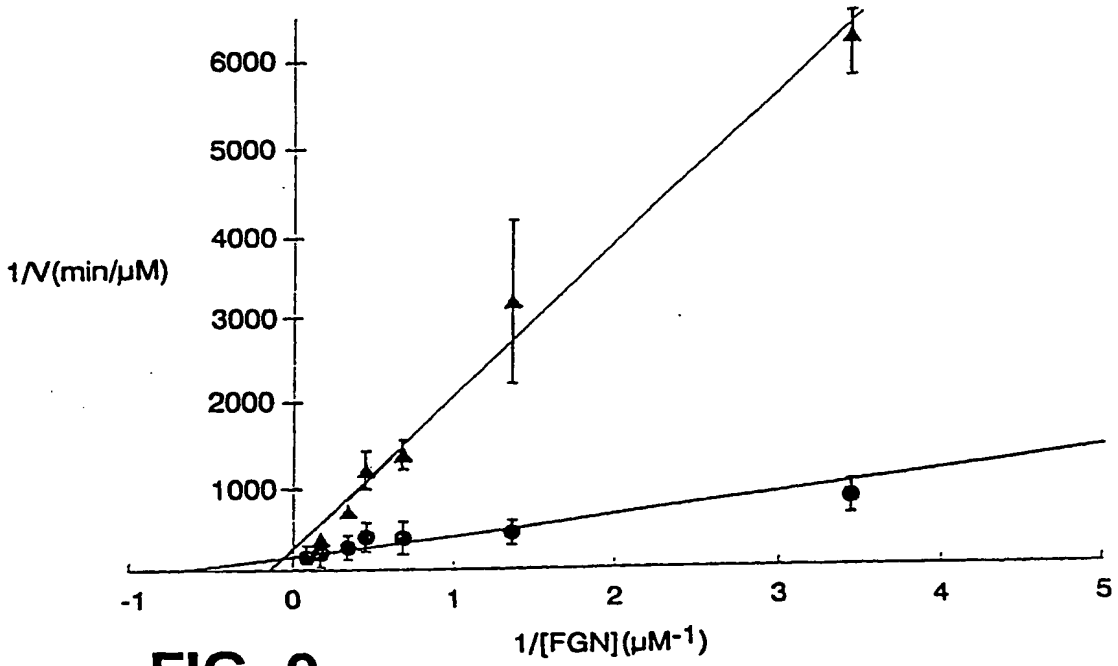

FIG. 9

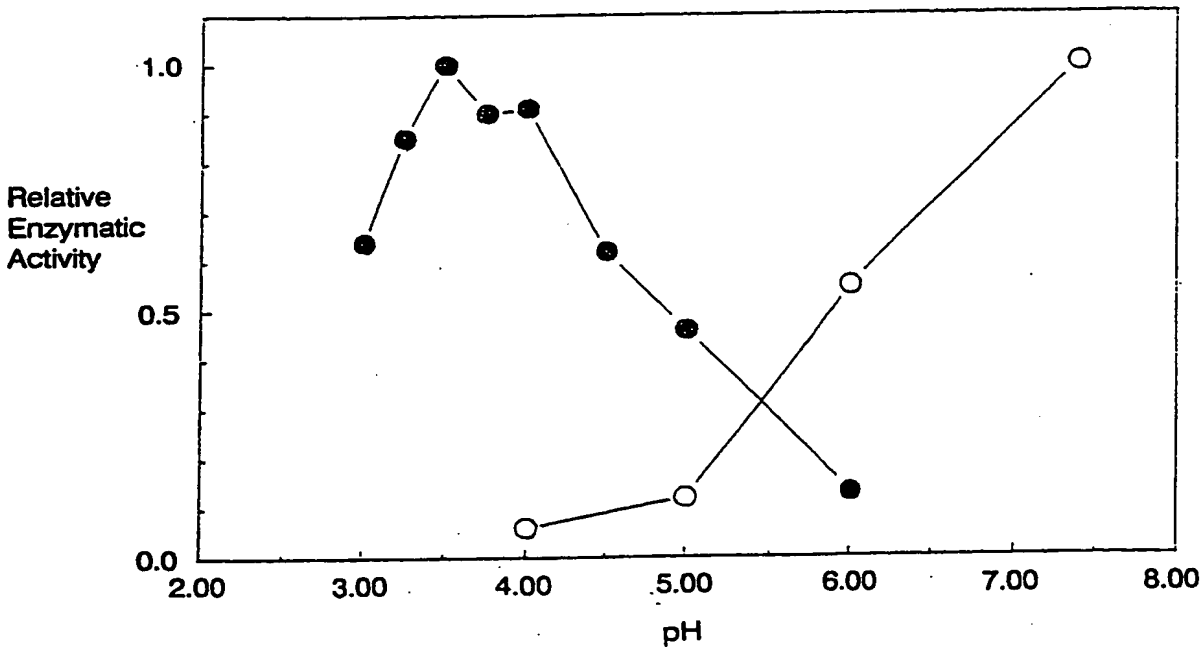

FIG. 10

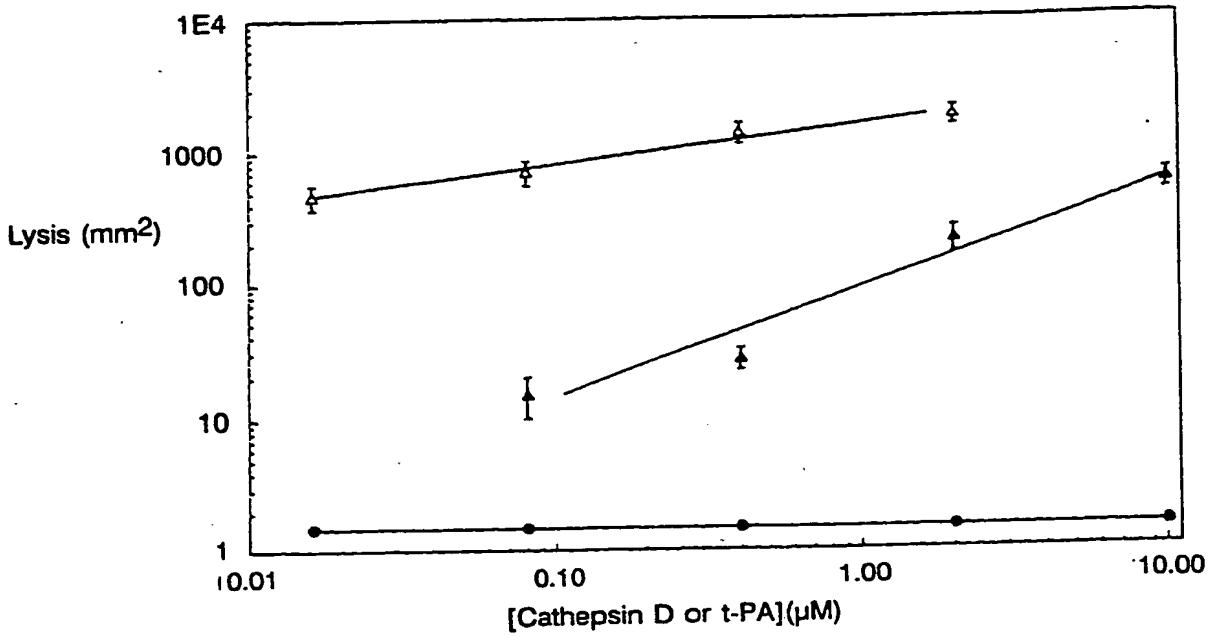

FIG. 11

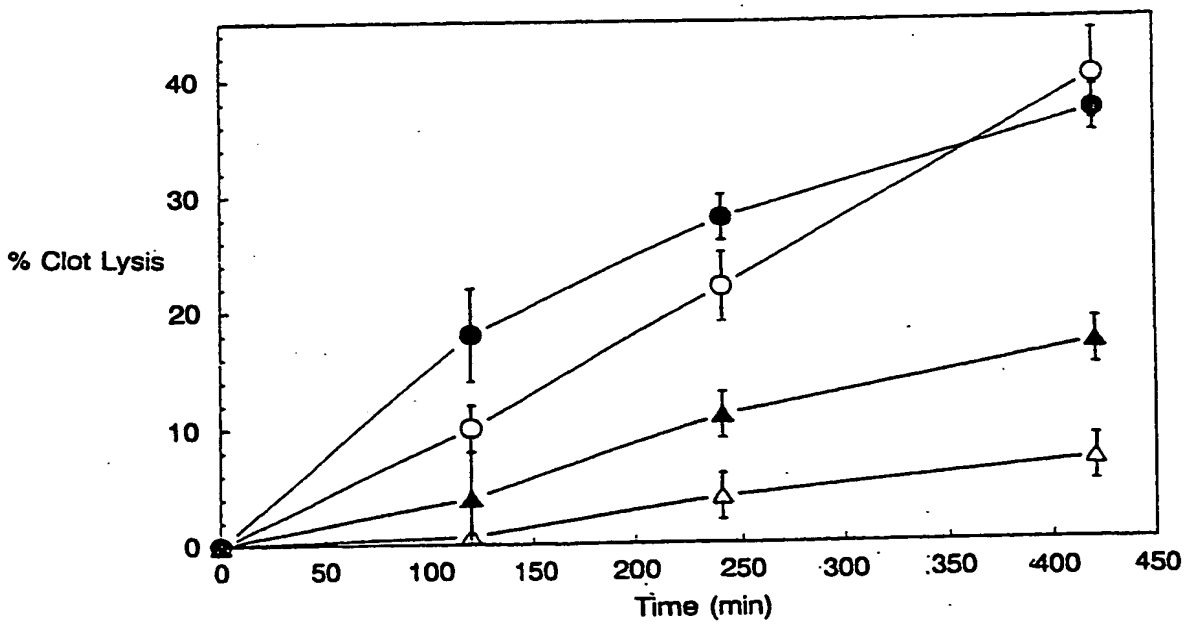

FIG. 12

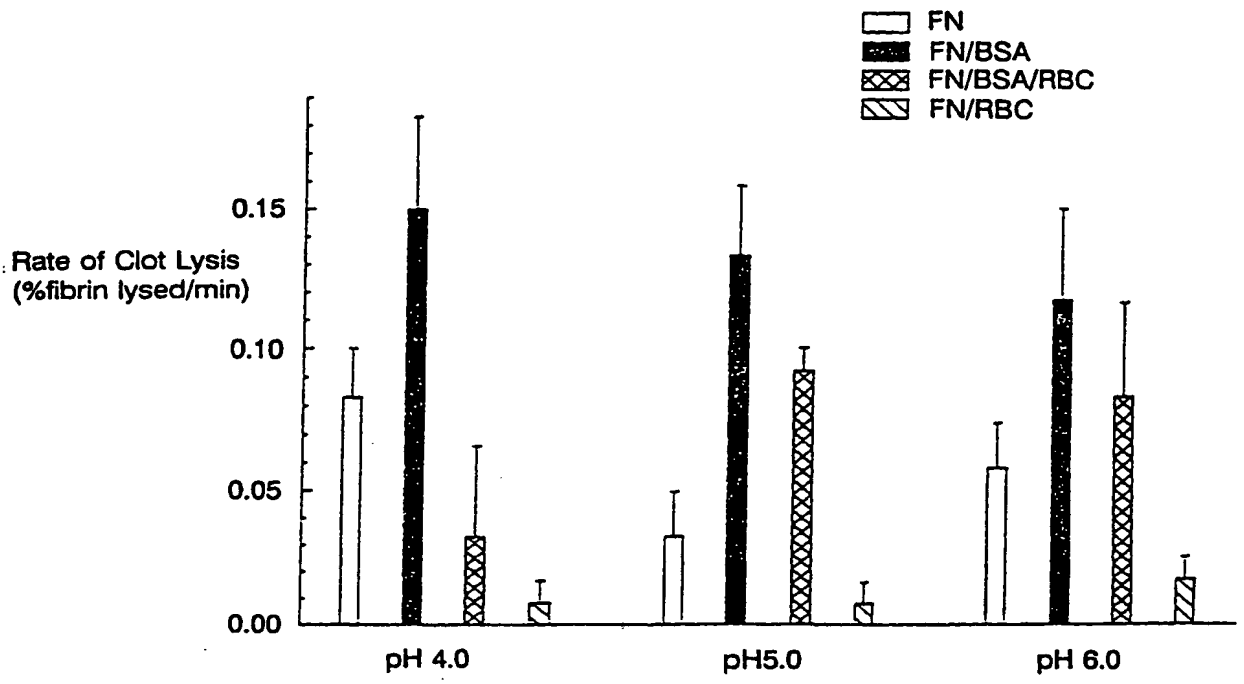

FIG. 13

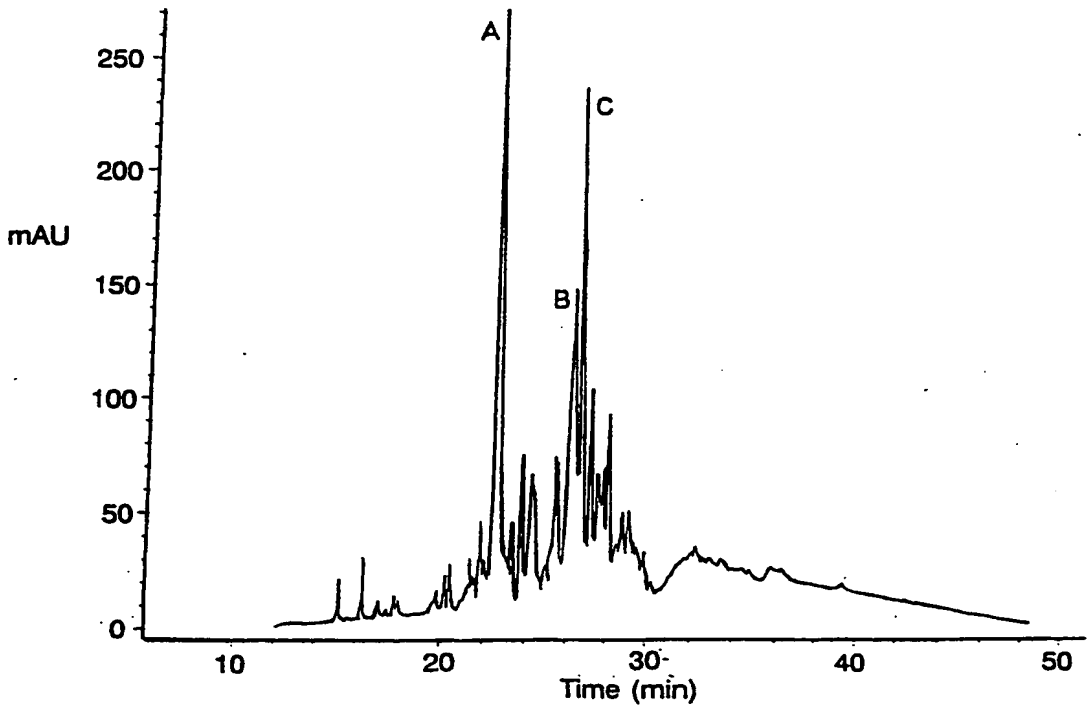

FIG. 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12583

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/46
US CL :424/94.66

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.66

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, EMBASE, MEDLINE, CA, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biol. Chem. Hoppe-Seyler, Volume 369 (Supplemental), issued May 1988, D. Gabrijelcic et al., "Proteolytic Cleavage of Human Fibrinogen by Cathepsin B", pages 287-292 see entire document.	1-8
Y	US, A, 3,973,001 (JAEGER ET AL) 03 August 1976, col. 3, lines 57-67.	1-8
Y	J. Clin. Invest., Volume 71, issued February 1983, D. Collen et al, "Thrombolysis with Human Extrinsic (Tissue-Type) Plasminogen Activator in Rabbits with Experimental Jugular Vein Thrombosis", pages 368-376, see entire document.	6, 7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 JANUARY 1995	Date of mailing of the international search report 13 FEB 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Kristin Larson Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Biochemical Society Transactions, Volume 13, No. 2, issued 1985, N. Pejhan et al, "Nature of a Fibrinolytic Acid Proteinase from Human Plasma", page 390.	1-8
A	Biochemical Society Transactions, Volume 13, No. 2, issued 1985, G.K. Watterson et al, "Assessment and Use of an Assay System for a Fibrinolytic Acid Proteinase from Human Plasma", page 391.	1-8
A	Biochemistry, Volume 29, No. 14, issued 1990, D.V. Brezniak et al, "Human alpha- to gamma-Thrombin Cleavage Occurs with Neutrophil Cathepsin G or Chymotrypsin while Fibrinogen Clotting Activity is Retained", pages 3536-3542.	1-8
A	US, A, 5,196,404 (MARAGANORE ET AL) 23 March 1993.	1-8
A	US, A, 3,646,195 (ERIKSSON ET AL) 29 February 1972.	1-8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12583

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-8

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

International application No.
PCT/US94/12583

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-8, drawn to a method of dissolving thrombi in blood.
II. Claims 9-11, drawn to a method of preventing unwanted bleeding.
III. Claims 12 and 13, drawn to a method for screening candidate compounds to identify a compound capable of inhibiting aspartyl protease-mediated fibrinolysis by monocytes.
The inventions listed as Groups I, II, and III do not meet the requirements for Unity of Invention for the following reasons:
The three groups are drawn to distinct methods which require different process steps and ingredients. Group I requires an aspartyl protease, Group II requires an inhibitor of aspartyl protease-mediated fibrinolysis by monocytes, and Group III requires a Mac-1 expressing activated monocyte and contains steps not required to practice the invention of Group I or II.
A reference which would anticipate the invention of one group would not necessarily anticipate or even make obvious any other group.
Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.